

The University of Chicago

THE RELATION OF NUMBERS OF
PARAMECIUM CAUDATUM TO
THEIR ABILITY TO WITHSTAND
HIGH TEMPERATURES

A PART OF A DISSERTATION SUBMITTED
TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

1933

By

MURVEL RILEY GARNER

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THE RELATION OF NUMBERS OF PARAMECIUM CAUDATUM TO THEIR ABILITY TO WITH- STAND HIGH TEMPERATURES¹

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THE possibility of beneficial effects of crowding of animals has been the subject of considerable recent experimental work. These effects may be of two sorts: first, those involving a differential in the rate of reproduction or growth; and, second, those which change the ability of the animals to survive unfavorable conditions. Robertson (1921a, 1921b, 1924a, 1924b) found that, under certain conditions grouped Infusoria reproduced more rapidly than isolated individuals. He assumed the presence of a substance given off by the animals during fission into the medium, which had an accelerative effect on the rate of reproduction. He proposed the term "allelocatalysis" for the phenomenon. Robertson's work has been variously confirmed and disputed by succeeding investigators. Allee (1931, 1934) has reviewed the literature dealing with this subject quite fully and critically.

In the matter of group protection against unfavorable conditions, Robertson (1924a), working with *Enchelys farcimen*, made the following observations with respect to numbers of animals in relation to resistance to high temperatures:

Temperatures above 30° C. prevent subcultures of single individuals of *Enchelys farcimen* from multiplying and the isolated individual at this temperature almost invariably dies. Cultures initially containing a large number of individuals, however, will multiply and survive continued exposure to considerably higher temperatures. Individuals isolated from such cultures and kept at the same temperature invariably die. Hence the contiguity of many organisms affords mutual protection against the adverse effects of high temperature.

Robertson assumed that the same substance which he postulated as the cause of acceleration in the reproductive rate of grouped animals over isolated ones served also to protect grouped animals against

¹ The writer acknowledges his gratitude to Dr. W. C. Allee for suggesting this problem and for giving aid and advice in its investigation.

high temperatures. There is no record that he went further in the analysis of this phenomenon. Drzewina and Bohn (1926, 1928) reported group protection of sperm against high temperature and also assumed the secretion of a protective substance.

The resistance of *Paramecium* to high temperatures has been studied by Hutchison (1913, 1915), who found that the resistance varied among races and in relation to the salt content of the medium; that the resistance was increased with an increase of the salt content of the medium; also, that heat resistance was not affected perceptibly by the age of the culture and its accompanying changes, nor was it affected by the ordinary changes in the acidity of the medium. Working with a more refined technique, Chalkley (1930), while investigating the mechanism of death in *Paramecium*, found a close correlation between the pH of the medium and the time required to kill the animals when suddenly exposed to a temperature of 40° C. He reported that the curve of resistance, with the pH varied, is bimodal, with a high resistance at pH 6.8, a maximum at pH 8.0, and a lesser resistance between these points, at pH 7.2. He also reported that an excess of sodium salts greatly reduced the resistance time; that an excess of potassium salts reduced the time in the acid range but increased it in the alkaline range; and that an excess of calcium salts increased the time in the acid range but reduced it in the alkaline range.

The conclusions of Hutchison and Chalkley, as noted above, were approximately diametrically opposed. It seems quite possible, however, that Hutchison's technique was not sufficiently refined.

In relation to varying temperatures a number of bimodal curves have been shown to exist. Heilbrunn (1924) found that the effect of temperature on the viscosity of protoplasm is expressed in this way. Earlier, Plough (1917) had found a similar situation with respect to the phenomenon of crossing-over in *Drosophila*. Brown and Banta (1932) found that a bimodal curve was required to express the effect of temperature on the production of males of *Moina macrocopa*.

MATERIALS AND GENERAL METHODS

Since a number of workers following Robertson had questioned both the phenomenon of the acceleration of the division rate of

groups of Protozoa over isolated individuals and also his explanation of it, it seemed advisable to reinvestigate the matter of group protection of Infusoria against lethal temperatures. *Paramecium caudatum* was chosen for this purpose. The animals were maintained in standard hay infusion. This was prepared by boiling 5 grams of timothy hay in a liter of tap water for 10 minutes and then straining the liquid through flannel. The infusions were inoculated with bacteria from previously made cultures. They were seeded with *Paramecium*, usually 4-6 days after the bacteria were introduced. The animals were maintained in as homogenetic a condition as possible by starting new isolation cultures from individuals taken at random from the clone. These re-isolations were made at intervals of not more than 2 weeks. In earlier experiments $\frac{1}{2}$ liter and 1-liter stock cultures were used, while in the later experiments 2-liter and 10-liter stock cultures were maintained. During the times of the year when these stock cultures could be maintained at little variation in temperature in the laboratory they were kept on the laboratory tables. For these, temperature variations were recorded. When, however, weather conditions became unsettled, the cultures were removed to inside rooms, where the temperature fluctuation did not exceed 3° or 4° C. In some of the later experiments the stock cultures were kept under constant temperature conditions. A few exceptions to this procedure will be pointed out later. In the experiments for maintaining high, constant temperatures there were used, at various times, two Freas water baths, two specially constructed constant-temperature rooms in Whitman Laboratory at the University of Chicago, and a specially devised water bath which maintained a constancy of temperature to within 0.15° C. The most critical of the experiments were performed using this apparatus. Finely drawn pipettes were used for handling the animals. Counts and other examinations were carried on by means of a binocular dissecting microscope. Depression slides of various capacities from $\frac{1}{2}$ to 2 cc. were used for isolation and small group cultures. These were kept in 9-inch covered glass dishes. Mass culture experiments were made in glass jars. The sizes most commonly employed were of 2-liter and 10-liter capacities. Ordinary bacteriological culture tubes were used to contain samples of large mass cultures in the later experiments where samples were

employed to ascertain conditions in large cultures. Populations of cultures were taken by direct count in the case of depression-slide cultures; they were approximated in the case of mass cultures by counting the populations of measured samples, a procedure employed and evaluated by Jahn (1928). In counting large numbers of individuals in a small area, they were drawn slowly into a capillary pipette and counted as they entered. This method was found to be satisfactory for sample counts but not for counts when the animals were to be used again, as usually some of the individuals would stick to the side of the pipette, so that the number expelled from the pipette would be considerably less than the number taken in. Records were kept of the age and pH of culture media and the age of clones used in the experiments. In the earlier experiments pH readings were taken by means of a Youden potentiometer. For the later ones, Hynson, Wescott, and Dunning and Hellige colorimetric sets were used.

EXPERIMENTS

A. RESISTANCE OF SMALL GROUPS AND ISOLATED ANIMALS

Robertson's original observations on the relation of age of culture to resistance to high temperatures had been with mass cultures as they had been kept in his laboratory during the extremely hot weather that sometimes prevailed. He found that sparsely populated (i.e., freshly seeded) cultures showed less resistance to heat than densely populated ones; also, that isolated individuals showed less resistance than members of dense populations. He did not record whether the isolations were made into the same medium in which the animals had been living or fresh medium. Few other data were recorded. Peterson and others who attempted to repeat other phases of Robertson's experiments usually used isolation or small group cultures in depression slides as a means of determining the behavior of individuals. In order to conform to this type of approach in the analysis of the resistance of *Paramecium* to high temperatures, the depression-slide technique was employed in the earlier experiments. The animals were placed in plate-glass slides which contained depressions of approximately 2 cc. capacity. In a single series five animals were placed in one slide with a given amount (usually 1 cc.) of infusion and five other animals were placed one each in five other

slides containing equivalent amounts of medium. The volume of medium, the kind and condition of medium, the treatment of the animals with respect to washing, and the temperature were varied in different experiments. The bacterial growth varied widely among the cultures of an experiment; and there were marked variations in the shape of the mass of medium on the depression slide. These affected the rate of evaporation and the ratio of glass surface in contact with the medium to the volume of the medium. The latter is about three hundred times as great in a depression slide as in a large stock culture. The glass surface ratio might affect the depression-slide cultures in two ways; first, by removing from the culture bacteria and bacterial products, as shown by the formation of a slime of zoögloea on the glass in contact with the medium; and second, perhaps, by throwing into the culture a solution of the glass substance. Many workers have shown that slime and mucous secretions affect media in which animals live by adsorbing certain substances from them. Carpenter (1927) showed that fishes can remove considerable quantities of lead nitrate from solution by adsorbing it in the mucus on their bodies. Allee and Bowen (1932) accounted quantitatively for the removal of colloidal silver from a suspension by goldfishes in a similar manner. It is impossible to say at present to what extent or in what way the zoögloea on a depression slide adsorbs any substance which might affect the resistance of *Paramecium* to high temperatures. With respect to the possibility of some of the glass substance becoming dissolved in the medium, see Kalmus (1929, 1931). The depression slides were made of hard plate glass and were thoroughly cleaned and soaked for long periods in distilled water as a means of removing all soluble substances. General observations indicate that the zoögloea relationship is more important in the present experiments than is the solution of materials from glass.

Later studies showed that the successional cycle in pH was carried as far in 12 hours in 1 cc. cultures as it was carried in 2 weeks in much larger cultures. The variable results noted in the foregoing experiments, due to the variable factors just given, were magnified still further by the action of the high temperature in speeding up the reproductive rates of both the *Paramecia* and the bacteria. Therefore,

this type of experiment was abandoned in favor of others in which the cultures developed more slowly and which could be more nearly completely controlled.

B. RESISTANCE IN MASS CULTURES

1. *During normal development of cultures.*—Jahn (1929) studied the reproductive rate of *Euglena* sp. in relation to the density of population of the culture. He used a mass-culture method. He estimated populations by counting the organisms in small samples from the cultures, as a substitute for the absolute counts of the numbers of organisms in depression-slide cultures, which had been used customarily. He believed that sample counts from large cultures gave a more accurate representation of the normal conditions of cultures than the counts from slides where the life-conditions are far from normal. Many other workers have pointed out that isolation and other small cultures on slides fail to give an accurate picture of the normal life-processes of microorganisms. In the course of the experiments described in the preceding section it was seen that both at high and at room temperatures the large stock cultures of *Paramecium* carried on much better than did cultures in depression slides. Mass cultures were frequently found with population densities as great as 500 animals per cc., a number that never was approached in 1 cc. cultures. Jones (1930) has made similar observations.

Chalkey (1930) removed small numbers of *Paramecia* from mass cultures and exposed them to lethal temperatures under varying conditions of pH as a method of studying the correlation between resistance to heat and the pH of the medium. His experiments suggested, as a means of determining the variations in heat susceptibility at the different stages of a culture cycle, the possibility of maintaining the organisms in large stock cultures at moderate temperatures, while checking their population changes by means of sample counts and measuring their resistance to high temperature through the use of sample animals taken from the large group.

After obtaining only negative results from depression-slide cultures, it was decided to attempt an adaptation of Jahn's and Chalkey's techniques for the study of the resistance to high temperature in *Paramecium*.

The initial experiments were carried out as follows: Several series of mass cultures were set up. A single series was made as follows: A 10-liter quantity of standard bacterized hay infusion was made and allowed to stand for 1-4 days. Individual cultures were made from this by straining the infusion through flannel and placing 2 liters in each of four 4-liter jars. In order to make the medium as nearly identical as possible in the four jars, it was stirred frequently during the measuring-out process. Animals were obtained for mass seeding from a rapidly dividing culture. They were washed by mild centrifuging, so that their original medium was diluted at least five hundred thousand times. Then they were further diluted with medium into which they were to be placed until they gave consistent population counts of approximately 100 per cc. One jar of each series was left unseeded to serve as a control for the normal bacterial effects; the other three were seeded with the freshly washed animals, so that they had an initial population of approximately 0.05 animals per cc., 0.2 animals per cc., 0.8 animals per cc., respectively. It is noted that each succeeding concentration is four times that of the culture immediately preceding.

Observations were made at intervals of 24 hours. The pH of the blank and the three inhabited jars was recorded. The media were stirred thoroughly, and 1-cc. samples were taken out for counting the population. With the population less than 50 per cc., ten 1-cc. samples were counted; with the population from 50 to 200 per cc., five counts were made; with the population over 200 per cc., three counts were made. The average of these counts was computed, and this figure was taken to represent the population density of the culture for that time. It is believed that this method gives a close approximation to the actual number of animals present.

In order to determine the resistance to heat, after considerable experimentation with other temperatures, a temperature of 40° C. was used. This temperature was found to have the advantages previously determined by Chalkley (1930), especially since the death time ranged from 10 to 20 minutes. The significance of this particular temperature will be discussed later. In making the resistance test, 1 cc. of the culture medium containing the average number of organisms was placed in each of five $\frac{1}{2}$ -inch bacteriological culture

tubes. These were placed, at 2-minute intervals, in a water bath heated to 40°. In the earlier experiments the tubes were paraffined as an additional precaution against the operation of any factors associated with the glass. Parallel tests with paraffined and unparaffined tubes soon showed that the practice of coating the tubes with paraffin made no difference in the resistance time and so it was abandoned. This finding coincides with that of Johnson (1933), who investigated the desirability of coating depression slides with paraffin in the study of reproductive rates in *Oxytricha*.

The tubes were taken out of the bath at the end of a period previously determined as one which would indicate the death time. They were then cooled for 20 minutes by standing in running water at a temperature of about 20° C. and poured onto depression slides for examination. The actual numbers of animals in the samples taken for the tests varied within the normal range for random sampling. Under some of the conditions of the experiments the animals disintegrated under the influence of the heat. These factors made it impossible to use the time of survival of 50 per cent of the organisms as an index of their heat-resisting ability, as would be customary in an experiment of this sort. Observations on the death time showed that only rarely did a particularly tenacious animal survive much longer than the rest of the group. Therefore, that exposure interval at which the last signs of life were detectable was taken as the index of heat resistance.

Table I gives a summary of the conditions of pH, density of population, and survival time against a temperature of 40° C. in relation to the age of the cultures, for one of five series run under comparable conditions. Examination of the data from these experiments permitted a number of generalizations which were useful in their further analysis.

As reported by many previous workers, a typical hay infusion has a pH value, when freshly made, of about 7.4. In about 2 days this value has dropped to around pH 6.0. Then the value rises through the next 3 or 4 days to the initial level. After this there is a further gradual increase to about pH 8.0, near which point the pH remains throughout the rest of the history of the culture. The exact values for the points on a curve plotted to show this trend vary with the

nature of the water used, with the size of the cultures, and probably with the nature of the bacterial flora. The pH cycles in cultures used in some of the later experiments which were made up from water much richer in calcium carbonate averaged about five-tenths of a pH unit higher at comparable stages in the progression. The cultures of one series were made from freshly boiled culture medium. The first part of these culture histories was passed through under conditions of low pH. The other series of cultures were inoculated after

TABLE I
HEAT RESISTANCE OF *Paramecium* IN RELATION TO DENSITY
OF POPULATION AND PH OF CULTURE

The "A" cultures were left unseeded with animals in order to show the normal pH cycle of cultures without Infusoria.

AGE OF CULTURE IN DAYS	SERIES X6										
	Population				pH				Resistance to 40° C. in Minutes		
	A	B	C	D	A	B	C	D	B	C	D
0.....	0	0.05	0.2	0.8	6.60	6.60	6.60	6.60			
1.....	0	0.4	1.3	5.5	7.15	7.11	7.11	7.11		20	20
2.....	0	4.2	10.7	47.5	7.10	7.11	7.20	7.20	21	21	21
3.....	0	27.4	73.8	210.0	6.94	7.10	7.26	7.26	16	14	13
4.....	0	120.3	212.0	431.3	6.99	7.20	7.26	7.24	15	15	14
5.....	0	402.7	415.3	436.3	7.26	7.37	7.37	7.33	11	10	10
6.....	0	451.0	428.0	436.3	7.34	7.37	7.45	7.45	15	16	18
7.....	0	465.0	426.0	443.5	7.61	7.53	7.59	7.59	22	21	22

the pH had dropped to the minimum and had started back to higher levels.

The pH cycle of the cultures runs through practically the same course and at the same rate, regardless of the density of the initial seeding with *Paramecia* or even the lack of seeding. Table II shows this relationship. These figures tend to contradict the conception expressed by Darby (1930) and others that protozoan populations affect materially the pH of their cultures. Darby, using *Paramecium aurelia*, *P. caudatum*, and *Stylonichia pustulata*, suggested that each organism tends to buffer its medium so as to bring it to its optimum pH, and that the more animals put in at a time the more quickly this

is brought about. It seems possible that in placing large numbers in the medium, as contrasted with small numbers, he may have carried over larger quantities of bacteria, so that the pH cycle which depends on bacterial growth naturally would be accelerated. The bearing of this question on the present problem will be discussed later in this paper.

The populations of the different cultures within a series were existing under quite similar environmental conditions at any given time. This was indicated by the close correspondence in pH values noted above, and by the fact that the populations of the cultures in a single series tended to reach approximately equal maximum concentrations

TABLE II
THE pH CYCLE IN UNPOPULATED MEDIUM COMPARED WITH
CYCLES IN POPULATED MEDIA
(Based on data of which those shown in Table I are typical.)

	CULTURE SERIES					
	X ₄	X ₅	X ₆	X ₇	X ₈	Total
Number of times blank culture has lower pH than the populated cultures of the same series	12	8	13	2	3	38
Number of times blank culture has same pH as the populated cultures of the same series. . . .	9	7	4	4	6	30
Number of times blank culture has higher pH than the populated cultures of the same series	6	0	3	13	6	28

irrespective of the density of the original seeding. Myers (1927) made similar observations with respect to population maxima in cultures of *Paramecium*.

The daily reproductive rate varied widely among the various cultures and in the various stages of the same culture. The daily reproductive rates may be expressed by "reproductive quotients," obtained by dividing the number of organisms in a unit volume of the culture on a given day by the number in a corresponding volume on the preceding day. This represents the average number of progeny of a single animal for the given period. The reproductive quotients in this series of experiments varied between 1 and 10. The highest values were found in the earlier stages of the cultures but

occurred in populations up to 200 animals per cc.; e.g., the quotient for the "A" culture in Series X₇ for the fifth day, where the population leaped from 29.9 to 203.7, is approximately 7.

In addition to the foregoing general observations based on data shown in part in Table I, the data were analyzed further to determine whether there was a correlation between the survival time of the organisms and any other recognizable factors. In order to group results into larger units, the survival time was classified as follows: 8-12 minutes were classified as "short time"; 13-16 as "medium time"; 17-21 as "long-time." On this basis the survival time was short in fourteen instances, medium in twenty-five instances, and

TABLE III
SURVIVAL TIME OF *Paramecium* AT TEMPERATURE OF 40° C. IN
RELATION TO AVERAGE REPRODUCTIVE QUOTIENTS
(Based on data of which those shown in Table I are typical.)

	No. of Instances	Average Quotient	Maximum Time in Minutes	Minimum Time in Minutes
Short time (8-12 min.)	14	4.4	10	1
Medium time (13-16 min.)	25	4.0	8	1
Long time (17-21 min.)	21	3.6	10	1

long in twenty-one instances. Table III gives these data in relation to the average reproductive quotients for each group.

On account of the extremely wide spread of the numbers from which the average quotients are computed, these figures have no statistical significance. Nevertheless, it is of interest to note that the longest time of heat resistance is associated with the smallest average quotient of reproduction. If, as is usually supposed, the fission rate is a measure of metabolic rate, then, according to the theories of Child (1915), the more slowly dividing animals would be expected to survive sudden exposure to relatively high temperatures longer than those which divide more rapidly. Although these figures are not statistically significant in themselves, they will be referred to later as indicating a possibly modifying effect on other factors.

In correlating the survival time with the population per cubic centimeter, it was difficult to establish a proper basis for grouping

populations. On a somewhat arbitrary basis, however, the populations were classified as "sparse" when the organisms numbered less than 10 per cc., "medium" when they ranged between 10 and 80 per cc., and "dense" when they ran over 80 per cc. Table IV shows this correlation. Here, again, the wide scatter of the numbers from which the averages are obtained, render them of little statistical significance except in the comparison between the sparse and dense populations, where the latter are shown to have a decidedly longer survival time.

A third type of correlation capable of analysis from such data as shown in part in Table I is that between the pH of the cultures and

TABLE IV
POPULATION PER CUBIC CENTIMETER OF CULTURES IN
RELATION TO SURVIVAL TIME
(Based on data of which those in Table I are typical.)

	No. of Instances	Average Time of Survival in Minutes
Sparse (less than 10 per cc.).....	19	12.5
Medium (10-80 per cc.).....	31	13.2
Dense (over 80 per cc.).....	32	16.0

the survival time of the populations. The survival times for the various pH values were averaged, with the results shown in Table V. It is seen that the curve of resistance is roughly bimodal. Chalkley (1930) found that the resistance to a temperature of 40° C. by *Paramecium caudatum* varied with the pH of the medium in which the animals were exposed; the curve here also was bimodal, with high points at pH 6.8 and 8.0. He also found that the loci of the curve were modified by excesses of various salts in the medium and that slight departures from normal in the chemical content of media exert strongly modifying effects on the resistance time of the animals. Since a curve based on present data would be still more of a departure from Chalkley's normal curve, it may be assumed that there are factors not yet analyzed that also modify the reaction.

Examination of Tables II, III, and IV shows that the conditions conducive to greatest survival are furnished by cultures which have

attained a dense population of animals with reproduction markedly slowed up, and which have progressed to a relatively high and stable pH.

The content of an old culture medium differs from that of a new one in several important respects: in the numbers and species of the bacteria, in the numbers of *Paramecia*, and in the excretion products of the bacteria and the *Paramecia*. Allee (1931) has shown, using *Procerodes*, in experiments to test survival to hypotonic sea water that the last animals to die do so under very different conditions from those under which the first animals died, and that they lived longer because animals had previously died in the medium. This suggests

TABLE V
RELATION BETWEEN pH OF CULTURE MEDIUM AND SURVIVAL
TIME OF *Paramecium*

pH of Culture	No. of Instances	Average Survival in Minutes	pH of Culture	No. of Instances	Average Survival in Minutes
6.5.....	4	10.5	7.4.....	4	15.7
6.6.....			7.5.....	18	15.9
6.7.....	4	13.5	7.6.....	1	11.0
6.8.....	5	14.0	7.7.....	3	17.7
6.9.....	4	12.5	7.8.....		
7.0.....	2	16.0	7.9.....		
7.1.....	10	19.9	8.0.....	3	9.0
7.2.....	11	13.8	8.1.....	3	9.0
7.3.....	11	10.0			

that there may have been a similar modification in some of the present experiments, especially those in which the survival time of very dense populations was tested. Certain of the experiments involved the survival time of more than 500 individuals in a single cc. of medium. Applying Allee's findings, it would seem almost certain that the last animals to die were present under highly modified conditions. Accordingly, a new series of experiments was set up designed to overcome this difficulty.

For the next set of experiments, cultures of 10 liters volume were used. These were seeded with approximately 0.1 animal per cc. Counts of the population were made as before. The pH readings were taken at the time of making the resistance tests. The procedure

for making the resistance test was to filter some of the medium to remove the animals, then to count back, by means of a capillary pipette, five animals into 1 cc. of the filtered medium. For a single determination of the resistance time ten to fifteen 1-cc. culture samples, each containing five animals were prepared and placed in the water bath as in the previous type of experiments. These were removed at intervals and placed on depression slides for counting. Counts were made under a binocular dissecting microscope. They were made continuously and as rapidly as possible during the time when the animals were dying. The samples were not set aside to cool, as in the preceding experiments, because it was believed that with continued practice dead animals could be distinguished from those merely rendered immobile by heat. In order to eliminate any error due to the fact that the last animals to die might live unusually long, it was decided arbitrarily to consider as the resistance time that in which only two of the animals survived. This time was established from at least three samples in each case, averages and interpolations being made when necessary. Table VI gives the result of the first of these experiments.

The population of this culture was below normal; the reproductive rate was unusually low throughout the entire period. The relationship between the pH value of the culture and the resistance time corresponds somewhat closely to that which Chalkley found, although the absolute values differ; this is due, in all probability, to differences in technique. That the difference in resistance time is not due to differences in numbers of organisms present is shown by the fact that during the last 5 or 6 days of the experiment there was little increase in the population. The culture showed an unusual variability in pH values, however, which is reflected in the resistance time.

2. *Under modified conditions.*—As a further means of testing the effect of pH, as distinguished from density of population, as the variable factor regulating the phenomenon, each day a 100 cc. sample was filtered out and acidulated with 0.1 N hydrochloric acid to pH values through which the culture had passed previously. No definite value was tried for at any time but a small amount of the dilute acid was added and the pH was tested. When a value was obtained that the culture had previously had, a small part of the sample was

set aside, and further acidulation was made. In this way two to four acidulated samples were prepared daily through most of the experiment. Then animals were washed once, and five were counted into 1-cc. amounts of each of the acidulated media. They were allowed to stand approximately 1 hour to acclimate to the changed pH and then were tested for heat resistance as in the case of the animals in the un-

TABLE VI

RESISTANCE OF *Paramecium* FROM 10-LITER CULTURE TO 40° C. WHEN EXPOSED IN GROUPS OF FIVE IN 1 CC. OF MEDIUM FROM THE CULTURE IN WHICH THEY HAD LIVED

Age of Culture	Temperature of Culture (Degrees Centigrade)	pH	Population per Cc.	Survival in Minutes
Beginning.....	24	6.8	0.1	
1 day.....	24	6.8	0.3	
2 days.....	24	6.8	1.5	8.5
3 days.....	24	6.9	7.1	9
4 days.....	24	7.1	19.6	8
5 days.....	24	7.2	30.3	7.5
6 days.....	24	7.5	39.7	12
7 days.....	24	7.6	38.6	13
8 days.....	24	7.7	40.1	14
9 days.....	24	7.7	43.6	15
10 days.....	24.5	7.8	45.2	16
11 days.....	24.5	7.9	52.0	17
12 days.....	24.5	7.8	59.4	16
13 days.....	24.7	8.0	62.2	21
14 days.....	25	8.0	62.8	19
15 days.....	25	8.1	65.1	18
16 days.....	25.2	8.2	66.5	15
17 days.....	25	8.0	66.3	19

modified medium. The results of this part of the experiment are given in Table VII. They show that resistance of animals in the later stages of a culture subjected to high temperature in their own medium which has been readjusted to the lower pH value of an earlier stage approximates that of animals that were present when the culture originally passed through that pH value.

Another experiment carried out immediately after the preceding one gave closely comparable results. These experiments indicate that under these conditions the pH of the culture exerts a greater in-

fluence on the survival time than does any direct effect of density of population.

In order to test further the effects of pH values, the density of population, and the changes in media due to having been inhabited, another experiment was performed. In the preceding experiment the water used had been Richmond, Indiana, city water, which was known to have a high calcium content. The calcium appeared to buffer the media to relatively high pH values. In order that the ef-

TABLE VII

COMPARISON OF THE TIME OF RESISTANCE OF *Paramecium* EXPOSED TO 40° C. IN UNMODIFIED AND IN REACIDULATED MEDIA FROM THEIR OWN CULTURE

DAY	PH	POPULATION PER CUBIC CENTIMETER	SURVIVAL IN MINUTES IN UNMODIFIED MEDIUM	SURVIVAL AT MODIFIED PH VALUES IN MINUTES									
				6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6	
4th.....	7.1	19.6	8	8	...	9							
5th.....	7.2	30.3	7.5	9	...	8	8						
6th.....	7.5	39.7	12	9	...				9				
7th.....	7.6	38.6	13	8.5	9			8		11			
8th.....	7.7	40.1	14	8	...				10	...	13		
9th.....	7.7	43.6	15			8				10			
10th.....	7.8	45.2	16	8.5	...					9.5	...	15	
11th.....	7.9	52.0	17					6			15		
12th.....	7.8	59.4	16		9							15	
13th.....	8.0	62.2	21				9		11		13		
Average survival time for modified pH values.....				8.8	9	8.3	8.5	7	10	10.2	13.7	15	

fects of the organisms, both bacteria and Protozoa, on the pH cycle might be, as far as possible, unhampered, a relatively unbuffered medium was sought. The medium used by Mr. Ralph B. Oesting, of the Zoölogy Department of the University of Chicago, as an artificial pond water for fish culture was found to be satisfactory, by using it in double concentration. As used in double strength, it is made by adding to each liter of distilled water, 0.4 mg. CaCl_2 , 0.2 mg. MgSO_4 , 17 H_2O , 0.2 mg. K_2SO_4 , and 0.2 mg. NaNO_3 . A 10-liter quantity of standard hay infusion was made, using the artificial pond water.

This had an initial pH of 6.2. From this, four 2-liter quantities were set aside in glass jars of approximately 4 liters capacity. These were designated as cultures "A," "B," "C," and "D." The "A" culture was left unseeded with *Paramecium* in order to check the normal pH cycle in an uninhabited culture. Cultures "B," "C," and "D" were seeded with *Paramecium* in the concentration of approximately 0.1 animal per cc. After seeding, these cultures were mixed together and re-separated into 2-liter amounts so that the initial bacterial and protozoan content might be as nearly identical as possible. The "B" culture was allowed to run through its cycle in a normal way. The "C" culture was allowed to run through its pH cycle until it came to pH 6.8. It was then held to that concentration by adding daily enough 0.1 N HCl to restore it to pH 6.8. The "D" culture was a reserve culture carried on under conditions like those of the "B" culture. Population counts were made daily from the "B" culture as described earlier in this paper. The "C" and "D" cultures had no population counts until the last day of the experiment, on account of lack of time. In maintaining the cultures the daily routine procedure was to take the pH of all the cultures, adjust the pH of the "C" culture to pH 6.8, make population counts from the "B" culture, and then run a series of resistance tests. These tests were made by using animals in groups of five as previously described. Animals were tested under various conditions as follows: animals from the "B" culture in their own filtered medium; animals from the "B" culture in medium filtered from the "C" (pH 6.8) culture; animals from the "B" culture in medium filtered from the uninhabited culture "A"; animals from the "C" culture in medium filtered from the "B" culture; and animals from the "C" culture in medium filtered from their own culture. When animals were tested in media other than their own, they were washed once in the medium into which they were being introduced, in accord with the method previously described and adopted as standard for this investigation, and then tested as quickly as possible. This procedure was adopted in an attempt to eliminate the possibility of acclimation and hereditary modifications of the sort which Jollos (1921) found to last through a number of fissions but which could not be assumed to be mutations. The data from these tests are expressed in Table VIII.

Various phases of this experiment serve to control each other, and they will be analyzed individually.

Comparison of lines (5), (6), and (8) shows that there is a marked correspondence in the pH cycles of cultures allowed to develop nor-

TABLE VIII

RESISTANCE OF *Paramecium* TO 40° C. UNDER CONTROLLED CONDITIONS
OF PH, DENSITY OF POPULATION, AND CONDITION OF MEDIUM
WITH RESPECT TO ITS HAVING BEEN INHABITED

"A" culture was unseeded. "B" culture was allowed to develop normally. "C" culture had pH adjusted daily to 6.8.

(1) Day of experiment.....	1	2	3	4	5	6	7	8
(2) Population of "B" culture per cc.....	0.1		1.6	10.2	49.7	261.0	354.7	360.0
(3) Population of "C" culture per cc.....	0.1							428.6
(4) Population of "D" culture per cc.....	0.1							313.7
(5) pH of "A" culture.....	5.7	6.8	7.0	7.2	7.25	7.3	7.4	7.4
(6) pH of "B" culture.....	5.7	6.7	7.0	7.2	7.25	7.3	7.45	7.7
(7) pH of "C" culture:								
Before adjustment.....	5.7	6.8	7.1	6.9	7.0	7.0	7.0	6.9
After adjustment.....			6.8	6.8	6.8	6.8	6.8	6.8
(8) pH of "D" culture.....	5.7	6.8	7.1	7.2	7.25	7.35	7.4	7.4
(9) Resistance of "B" animals in own medium.....			13	13.5	14	14.5	10	{*20 18
(10) Resistance of "B" animals in "A" medium.....			13	14.5	16	19	11	{*23 18
(11) Resistance of "B" animals in "C" medium.....			10	12	12	13	10	*11
(12) Resistance of "C" animals in own medium.....			13	13.5	12	11	10	15
(13) Resistance of "C" animals in "B" medium.....			13	13	11	9.5	10	†11

* Indicates substitution of animals from "D," another culture comparable to "B," because conjugation was observed in "B."

† Indicates substitution of medium from culture "D" for that of "B."

mally. An exception to this is to be seen in the 8th-day pH value in line (6). On the 7th day a number of conjugating pairs of animals were seen in the "B" culture. No suggestion is offered by way of correlating these two facts. The daily drive toward neutrality in the "C" culture which was readjusted daily to pH 6.8 was quite con-

stant in its magnitude; the value was either 0.1 or 0.2 pH points in every case.

A slight advantage to the animals in holding the pH at the constant figure of 6.8 over allowing it to develop normally is suggested, as is seen by comparing line (3) with (2) and (4). It was noted during the last 2 days of the experiment that the animals in the "C" culture appeared more plump and more active. This suggests, at least in certain aspects, that the high pH characteristic of old cultures is relatively detrimental to the animals populating them.

With respect to the time of resistance to high temperature a number of generalizations can be made. By comparing (9) and (10) it is seen that under the conditions of sudden exposure to high temperature previously uninhabited medium of an equivalent pH tends to be more satisfactory for the animals than their own medium which has been affected by their own presence. In applying "Student's" formula for testing the statistical significance of small numbers of paired experiments to the figures in lines (9) and (10), there is obtained a probability of 0.0466, which approaches statistical significance. By comparing the successive resistance times as shown in lines (9) and (10), it is seen that there is a tendency for the resistance times to increase day by day. This is correlated with two variables—the increase in population and the increase in pH value. However, since the animals live fully as long or longer in uninhabited medium than in that which has contained these organisms, it appears again that pH is the more significant factor. It should be recalled that the seventh-day readings in lines (9), (10), and (11) involve animals which were undergoing conjugation. It is not known what the cause-and-effect relationships are here; however, the decided drop from the previous day in each case implies a changed physiological state in connection with conjugation. That the phenomenon is a real one and not due to the accident of selecting abnormal animals for the test is indicated by the fact that these parts of the experiment were repeated twice immediately after the initial test, with identical results. The resistance of animals kept constantly at or near pH 6.8 does not undergo the same type of daily increase as does that in the cases where the pH tends to rise. The reading for the last day, however, for the animals kept constantly at pH 6.8 and tested in their

own media (line 12) is not easily explained on these grounds. Another part of the experiment which does not bear out the hypothesis that the important factor is pH is that in which the animals from the constant pH 6.8 culture were tested in medium from the inhabited culture undergoing normal pH changes (line 13). It should be borne in mind, however, that the tests were made as soon as possible after the counting-out process was completed, so that the results may express the condition of the animals rather than the condition of the culture.

To summarize the results of this experiment: The animals tend to increase their resistance to sudden exposure to heat with the increase in population and the rise in pH. Animals kept at a constant pH value tend to maintain a more nearly constant resistance time to 40° C., despite the fact that the population is rapidly increasing. For animals living in normally changing medium with respect to pH, there are indications that uninhabited medium of equivalent pH results in greater resistance time than their own medium, indicating that the changes produced by the animals themselves do not necessarily have autoprotective results and, in fact, may be harmful. Following this reasoning, then, it appears that the pH changes in a culture alone may account for the fact that densely populated cultures are more resistant to heat than new and isolation cultures.

DISCUSSION

The problem of the resistance of Protozoa to high temperature is not a simple one. Perhaps no other environmental condition than heat has more accompanying variable factors. In the use of depression-slide cultures at temperatures which are lethal only after some hours, the culture medium becomes radically changed from the initial condition. Evaporation of the medium takes place even in almost saturated atmospheres, at times until the culture has only a fraction of its original volume. This implies, of course, a corresponding concentration of the substances dissolved in the medium. Since Chalkley has shown that excesses of some salts affect the death time of Paramecia under high temperature conditions, evaporation must, at times, have marked consequences. Also, high temperature tends to drive out the dissolved gases, giving another type of radical

change. Heat greatly accelerates bacterial growth, and ever so slight initial differences in bacterial content of cultures become highly magnified with the passage of time. Correlated with bacterial growth, pH changes are rapid and drastic in small cultures. Jennings (1913) and others have called attention to the fact that the existence of Protozoa on depression slides is precarious.

On account of these difficulties, the depression-slide method of testing the heat resistance at barely lethal temperatures, of *Paramecia* is felt to be unreliable. The use of mass cultures eliminates, to a degree, the differences in environmental conditions of the organisms.

The temperature of 40° C. was selected for the mass-culture experiments because of the short time required for making the survival tests, thus doing away with many confusing changes in the medium. Experiments showed that animals which die in 15 minutes at 40° C. may live 1½ hours at 38° C., and some will live for as much as 6 days at 34° C. if mass cultures are used. The question arises at once as to whether a high degree of resistance to death at 40° C. where the death time is a matter of some 10–20 minutes is correlated with resistance at a lower temperature of, say, 34° C., at which temperature, as has just been stated, the same animals would live about 6 days. The data in this investigation do not answer this question directly, and further research is needed to determine the extent of this correlation, if any exists. However, the differences in death time at 40° C. imply variations in the physiological states in the animals under various conditions.

Robertson's original observations on the advantage of mass cultures over isolation cultures for the survival of Protozoa in high temperatures were made in his laboratory in the course of the hot season of the year. The probability is that, on the whole, the temperature rose gradually. This raises the question whether from the large numbers of individuals in mass cultures some might have developed an acclimatization to the changed condition while others perished, and whether the race might have become more resistant through the reproduction of these selected individuals. The inheritance of acclimatization characteristics has been reported frequently for Protozoa. Dallinger (1887; cited by Jennings, 1929) cultivated three

species of flagellates under conditions of very gradually increasing temperatures until the organisms were able to thrive at a temperature of 70° C. Ritz (1916) found that, as antibodies of the host act on trypanosomes, most of the organisms are killed, but that those that survive develop a resistant strain. However, Jollos (1921) practiced selection of resistant individuals of *Paramecium* to heat but found no hereditary diversities within a single clone. This coincides with Jennings' findings (1929) that within a single clone the animals are remarkably constant in their hereditary characteristics. If it can be assumed that Robertson's organisms were from a single clone, the possibility of hereditary acclimation characteristics would seem scarcely sufficient to account for his observed phenomenon.

Another possible source of difference in response in Protozoa when the small numbers of isolation and small group cultures are studied is the difference in time since the last fission. Some observations based on very few instances have indicated that animals in fission are more than usually resistant to sudden increase in temperature, while conjugation pairs are highly susceptible to heat. From this one would infer, as might be expected, that there are changes in physiological states during the interfission and interconjugation periods.

No attempt was made to study the various effects of different species of bacteria in the cultures at any given time or of bacterial succession. It is possible that bacteria play an important rôle in the phenomenon under consideration; the investigation of this question is, however, reserved for the present.

Since the data in this investigation indicate that the pH of the culture medium plays a large part in determining the resistance to high temperature of *Paramecium*, the question should be considered whether the animals aid in bringing about the pH changes. It has been noted that the usual course in a culture is toward an increased pH value while the numbers of animals are increasing. This increase in pH value frequently results in increasing the resistance of the animals to heat. If it can be shown, as Darby suggested, that the animals actually do aid in adjusting the pH to an optimum, a case would be made for group protection of a sort implied in Robertson's extension of his theory of allocatalysis. However, the experiments

here cited indicate that bacterial cultures run their pH cycle in a practically identical manner, regardless of whether *Paramecium* are present. Chalkley found that pH values above 8.0 have a harmful effect in that they shorten the resistance time of *Paramecium* to high temperatures. In these experiments this same tendency was indicated. Losina-Losinsky (1931) stated that Paramecia rapidly lose the ability to ingest bacteria as the pH increases above 8.0. In many cases the *Paramecium* cultures reach a pH stability in this high range.

In order to see whether some other factor than the presence of *Paramecium* in cultures affected the pH, a series of depression-slide cultures of various sizes were made, using freshly boiled and cooled,

TABLE IX
EFFECT OF SIZE OF CULTURE ON RATE OF PH CYCLE

Cultures are made from a 10-liter volume of freshly boiled and cooled, bacterized medium. The water is Oesting's artificial pond water.

No. of Cultures for Each Reading	Size of Culture	pH at Beginning	pH at 16 Hours	pH at 24 Hours
32.....	0.1 cc.	6.2	6.8	7.5
20.....	0.25 cc.	6.2	6.6	7.5
10.....	1.0 cc.	6.2	6.1	7.3
1.....	10 liters	6.2	5.7	5.7

bacterized standard hay infusion. These were made in the following numbers and sizes: sixty-four 0.1-cc. cultures in small depression slides; forty 0.25-cc. cultures in depression slides; twenty 1-cc. cultures in large depression slides of 2 cc. capacity each. The original 10-liter volume of infusion served as a control. The composite pH of half the cultures of each size group was taken at the end of a 16-hour period. The composite pH of the other half of each size group was taken at the end of 24 hours. Table IX summarizes these readings. These data show a marked inverse correlation between the size of the culture and the rate of the pH cycle.

Recalling Robertson's observations which motivated this investigation—namely, that single individuals die when isolated from stock cultures under conditions of the high temperatures found at times in his laboratory, while the stock cultures continue to live—this ob-

servation has been verified and the following explanations are suggested:

If the cultures used by Robertson did not arise from a single infusion, variations might be expected which would allow a greater number of heat-resistant forms to be found in the mass cultures than among the relatively few isolated animals. If Robertson's animals did come from a single clone, variations in physiological states such as accompany the fission cycle might favor the survival of animals in the mass cultures, or some of them, as compared with the fewer number isolated. But entirely aside from these possibilities, an explanation may be found in the relation between pH of the medium and resistance to heat—at least in the case of *Paramecium caudatum*.

An isolation culture is usually made by taking an animal from a stock culture of some considerable size and placing it in a depression slide with a small amount of fresh medium. The pH in these small cultures is rapidly carried into a range which decreases the resistance of the animal to heat. Chalkley (1930) pointed out three ranges of low resistance: below pH 6.7, around 7.2, and above 8.2. On the other hand, the stock culture has a retarded pH cycle which moves slowly through the range of greatest resistance—pH 7.4 to 8.0. Thus, pH effects alone seem sufficient to account for the observed phenomenon without the aid of Robertson's extension of his allelocatalytic hypothesis.

SUMMARY

1. Robertson (1924b), using *Enchelys farcimen*, observed that mass cultures withstand high temperatures better than do isolation cultures. He postulated an "allelocatalytic" substance given off by the animals which serves for mutual protection. This phenomenon has been re-examined and verified under certain conditions, without, however, verifying Robertson's explanation.

2. The isolation-culture method of testing the resistance of Protozoa to high temperatures was found to be impracticable because of the difficulty of controlling, at the temperatures used, evaporation rate, salt, dissolved gas and bacterial content, and pH value of small cultures.

3. A mass-culture method was developed; the cultures were held at room temperatures and sample individuals were taken out and tested under various conditions.

4. After experimentation, a temperature of 40° C. was adopted as a testing temperature for time of resistance of the organisms because it usually brought about death in from 10 to 20 minutes and thereby eliminated uncontrollable factors inherent in tests requiring much longer time.

5. The general conditions of greatest resistance to a sudden exposure to a temperature of 40° C. were those furnished by mass cultures which had become thickly populated and had reached a relatively high pH value. The relative importance of density of population and pH of the culture medium was studied, and it was found that pH is the more important in determining resistance time.

6. *Paramecia* do not affect materially the pH cycles of cultures.

7. In the normal course of pH cycles, cultures pass through two or three of the pH ranges where the resistance of the animals to heat is low. These ranges lie around the pH values of 5.7, 7.2, and 8.3, with ranges of greater resistance between, i.e., around pH 6.8 and 7.8 (cf. Chalkley, 1930). Chalkley reports that these ranges may be modified by excess amounts of certain salts.

8. The rate of change of pH of bacterized cultures within the limits tested is inversely proportional to the size of the culture. The difference in rate between depression-slide and 10-liter mass cultures is recorded.

9. It is recognized that a possible factor in the greater resistance to high temperatures of *Paramecia* from mass cultures is the greater number of animals present, with a correspondingly greater diversity of resistance abilities; and that the survival may be due to selection among these diverse forms.

10. It is suggested that the pH relationship alone seems sufficient to account for the observed phenomenon of the greater resistance to high temperatures with mass cultures than with isolation cultures among Protozoa, without the aid of Robertson's extension of his allelocatalytic hypothesis.

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